Anion Channels and Transporters

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Calcium-Calmodulin does not Alter the Anion Permeability of the TMEM16A Calcium-Activated Chloride Channel

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The binding of calcium-calmodulin (CaCaM) to various cation channels has been found to modulate the channel function. Recently, the anion permeability of the TMEM16A calcium-activated chloride channel was also reported to be altered upon modulation by CaCaM (Jung et al., *Proc Natl Aca Sci*: 110: 360-365, 2013). This modulation effect could be physiologically important for many cellular functions that involve the TMEM16A channel. To study the modulation of TMEM16A by CaCaM, we expressed the TMEM16A channel in HEK 293 cells and performed excised inside-out patch recordings. The anion permeability ratios were evaluated based on the reversal potentials in bi-ionic conditions in which various anions were placed in the intracellular side with an equal concentration of chloride ions in the extracellular solution. We used both the voltage-clamp and the current-clamp (I=0) recording approaches, and surprisingly found that CaCaM, when applied to the intracellular side of the channel, did not alter reversal potentials. The activity of CaCaM was verified by a positive control experiment in which the current of the olfactory cyclic nucleotide-gated channel encoded by CNGA2 was inhibited upon the application of intracellular CaCaM. These results contradict the recent finding that intracellular CaCaM alters the anion permeability of the TMEM16A channel. Our experiments also show that the measurement of reversal potentials is imprecise if the recorded current is > 1-2 nA at +40 mV, likely due to a series-resistance problem. We notice that the reversal potential change in Jung et al is particularly prominent at high intracellular [Ca^{2+}], which activates huge calcium-activated currents (>5-10 nA) in whole-cell configurations. Whether this technical issue causes the change of the reversal potential in Jung et al. requires further scrutiny.

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Serine 550 is Involved in the Regulation of Mouse TMEM16A-Caccs by CaMKII

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Ca^{2+}-activated Cl^- channels (CaCCs) recorded from vascular smooth muscle cells (VSMCs) are regulated by phosphorylation involving CaMKII, Calcineurin and PP1 (Ayon *et al., J Biol Chem* 2009;**284**:32507-32521). TMEM16A, a recently identified anion channel gene, is believed to encode for CaCCs in VSMCs (Davis *et al. Am J Physiol Cell Physiol* 2010;**299**:C948-C959; Manoury *et al. J Physiol* 2010;**588**:2305-2314) and to contribute to vascular tone (Davis *et al. Br J Pharmacol* 2013;**168**:773-784). This study aimed to determine whether Ca^{2+}-activated Cl^- currents (I_{ClCa}) generated by transient expression of mouse TMEM16A in HEK-293 cells are regulated by kinase and phosphatase activity. Immunocytochemical experiments confirmed the presence of PP1α, PP1β/δ, PP1γ, PP2A and CaMKII in HEK-293 cells, and mRNA transcripts for all four known mammalian CaMKII isoforms (CaMKIIα, β, γ and δ) were identified by RT-PCR in these cells. Similar to VSMCs, TMEM16A-induced I_{ClCa} displayed significant rundown in cells dialyzed with 5mM ATP; this effect was potently inhibited by removing ATP. The attenuation of TMEM16A-I_{ClCa} rundown in the absence of ATP was suppressed by intracellular application of the PP1/PP2A inhibitor okadaic acid (30 nM). Intracellular application of KN-93 (10 μM) or autocamtide-2-related inhibitory peptide (ARIP; 5 μM), two specific CaMKII inhibitors led to a significant attenuation of TMEM16A-I_{ClCa} rundown in the presence of ATP. While mutating a threonine 623 of TMEM16A, a putative CaMKII phosphorylation site, to an alanine did not affect rundown, a serine to alanine mutation at position 550 attenuated $I_{Cl(Ca)}$ rundown to an extent similar to that produced by KN-93. These data suggest that TMEM16A-I_{ClCa} is regulated by CaMKII and PP1 in a manner similar to CaCCs in VSMCs. Our data also suggest that serine 550 is an important contributor to the regulation of $I_{ClCa}$by CaMKII.

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Inhibitory Role of PIP2 on Calcium-Activated Chloride Channel Activity

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Ca^{2+}-activated Cl^- channels (CaCCs) in vascular and many non-vascular smooth muscle cells are thought to be encoded by the gene TMEM16A. Our group recently showed that the amplitude and pharmacology of CaCCs currents (I_{Clca}) are influenced by cholesterol depletion (Sones *et al. Cardiovasc Res* 2010;**87**:476-484). The aim of this study was to investigate the mechanism by which native CaCCs are regulated by cholesterol depletion and the possible role of phosphatidylinositol 4, 5-bisphosphate (PIP_2). Freshly isolated pulmonary artery myocytes were obtained from male Wistar rats (200-225g), sacrificed in accordance with Schedule 1 of the United Kingdom Animals Act (1986). In whole cell patch clamp recording mode, macroscopic I_{Clca} were elicited by dialysing the cells with a pipette solution set to 500nM 'free' [Ca^{2+}]. Single channel inside-out recordings were also induced by 500nM [Ca^{2+}] and displayed similar characteristics to earlier characterisation. These currents displayed the distinct voltage- and time-dependent properties described in previous studies. Cholesterol depletion from the cell membrane through the application of 3mg.ml^{-1} methyl-β-cyclodextrin (mβcd) augmented I_{Clca}, which was prevented by application of the TMEM16A specific inhibitor $T16A_{inh}$-A01. Augmentation by mβcd was also sensitive to the phospholipase C inhibitor U73312, but not the inactive analogue U73343. Inhibiting PIP_2 synthesis by incubation with 20μM wortmannin, a PI-4 kinase inhibitor augmented I_{Clca} and prevented the stimulatory effects of mβcd. Enrichment of the pipette solution with 1μM diC-8 PIP_2 attenuated $I_{Clca}$and suppressed the effect of mβcd. Wortmannin and PIP_2 effects on I_{Clca} were replicated in single channel inside-out recordings. Furthermore, the application of the poly-cation Poly-L-lysine (50μg/ml), which scavenges PIP_2, augmented $I_{Clca.}$ This study suggests that cholesterol depletion of the cell membrane increases TMEM16A/CaCC activity, partly through the removal of the inhibitory effect of PIP_2.

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Probing the Transmembrane Topology of TMEM16A(A)/Anoctamin-1 by Cysteine Scanning Mutagenesis

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TMEM16A(a)/Anoctamin-1 (Ano1) is a calcium-activated chloride channel. Using blue native polyacrylamide gel electrophoresis (BN-PAGE) and chemical cross-linking, we have demonstrated that the recombinantly expressed mouse Ano1 (mAno1) assembles as an obligate homodimer in both *Xenopus laevis* oocytes and HEK293 cells (Fallah et al Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.004697, 1-10, 2011). Here we replaced individual residues by cysteines in the four putative mAno1 ectodomains, whose boundaries were based on the predictions of the MEMSAT3 topology program (Jones, Bioinformatics 23, 538-544. 2007). As a quantifiable readout we examined the accessibility of the introduced cysteine residue at the extracellular membrane surface of intact *X. laevis* oocytes for a thiol-reactive fluorescent maleimide dye. The mAno1 mutants were purified by Ni-NTA affinity chromatography, resolved by BN-PAGE and SDS-PAGE, and visualized by scanning of the metabolically incorporated [^{35}S]methionine and the thiol-bound dye fluorescence. All the mutants examined appeared in the oocyte plasma membrane in the homodimeric state. Based on a total of 42 mAno1 cysteine mutants analyzed so far, the experimentally determined boundaries of the four mAno1 ectodomains (EC1-EC4) in terms of the residues positions are: 358-406 (EC1; predicted 359-402), 511-531 (EC2, predicted 513-527), 598-630 (EC3, predicted 598-707) and 791-848 (EC4, predicted 790-748). The major deviation pertains to EC3, which according to cysteine accessibility is much shorter, comprising residues 598-629 instead of residues 598-707 predicted by the MEMSAT3 program. Our data suggest that TM6 is represented by residues 630-650 (and not residues 708-732 predicted by MEMSAT3). In contrast, the predicted position of TM7 (residues 765-789) is in line with our experimental data. Our data confirm and extent earlier findings of Yu, Duran, Qu, Cui and Hartzell (Circ. Res. 110: 990-999, 2012).